

## Chemical Constituents of Malaysian *Apis mellifera* Propolis (Juzuk Kimia Propolis *Apis mellifera* Malaysia)

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### ABSTRACT

*Propolis is a resinous material collected by honey bees from various plant sources. The chemical constituents differ depending on the source of vegetation area. To date, no study of the chemical constituents of Apis mellifera propolis from Malaysia has been reported. Thus, the aim of this study was to determine the chemical constituents of Malaysian Apis mellifera propolis. Raw propolis samples were collected from Acacia mangium apiary from northern and southern regions of Peninsular Malaysia and were extracted using 70% ethanol. Then, the ethanolic extracts were evaporated and were lyophilized to dryness. The silylated compounds were identified by gas chromatography with mass spectrometry analysis (GC-MS). The compounds were characterised by comparison with NIST02 and Wiley275 libraries. Twenty-six individual compounds were identified and  $\beta$ -panasinsene was identified for the first time in propolis. The main compounds identified from both southern and northern regions of Peninsular Malaysia were terpenoids. Terpenoids belong to an important class in the propolis compounds due to its valuable biological properties. The amount of terpenoids ranged from 7.19 to 11.46% indicating that although the main plant source for both regions was Acacia mangium, other shrubs and fruit orchards surrounding the two apiaries may differ which may contribute to the variations.*

*Keywords: Ethanolic extract; GC-MS; Malaysian propolis*

### ABSTRAK

*Propolis adalah bahan resin yang dipungut oleh lebah madu daripada pelbagai sumber tumbuh-tumbuhan. Juzuk kimia berbeza bergantung kepada sumber kawasan tumbuh-tumbuhan. Sehingga kini, tiada kajian juzuk kimia propolis Apis mellifera dari Malaysia dilaporkan. Justeru, tujuan kajian ini adalah untuk mengenal pasti kandungan kimia propolis Apis mellifera Malaysia. Sampel propolis mentah telah dikumpul dari tempat ternakan lebah Acacia mangium dari kawasan utara dan selatan Semenanjung Malaysia dan telah diekstrak menggunakan 70% etanol. Kemudian, ekstrak etanol telah disejat dan dibeku keringkan. Sebatian sililasi telah dikenal pasti menggunakan analisis kromatografi gas dan jisim spektrometri (GC-MS). Sebatian dicirikan melalui perbandingan dengan perpustakaan NIST02 and Wiley275. Dua puluh enam sebatian individu telah dikenal pasti dan  $\beta$ -panasinsene telah dikenal pasti buat kali pertama daripada propolis. Sebatian utama yang dikenal pasti daripada kedua-dua kawasan selatan dan utara Semenanjung Malaysia adalah terpenoid. Jumlah terpenoid antara 7.19-11.46% menunjukkan bahawa walaupun sumber tumbuhan utama untuk kedua-dua kawasan adalah Acacia mangium, tetapi pokok renek lain dan kebun buah-buahan di sekitar dua apiari mungkin berbeza dan menyumbang kepada perbezaan.*

*Kata kunci: Ekstrak etanol; GC-MS; propolis Malaysia*

### INTRODUCTION

Propolis is one of the bee products consists of a mixture of resinous substances collected by honey bees from various plant sources, mainly from flowers and leaf buds and mixed with their saliva (Bankova et al. 2000; Marcucci 1995).

It is used to patch small holes in the beehives, to strengthen the borders of combs, to line all cells inside and balancing humid conditions or extreme drought. In addition, propolis is also used to embalm dead animals or insects that cannot be expelled by the honey bees (Ghisalberti 1979). Propolis comes from the ancient Greek words; “*pro*” and “*polis*”. *Pro* is before and *polis* is city, meaning before the city or defender of the city. Propolis means a substance to defence the beehive. Propolis has

been widely used in ancient remedies as early as 300 BC due to its various biological properties.

Propolis generally contains 45-55% resins which comprise of flavonoids, phenolic acids and their esters, 25-35% waxes and fatty acids, 10% essential oils (volatiles), 5% pollens and 5% minerals (Krell 1996). More than 300 chemical compounds were identified so far (Huang et al. 2014).

Chemical compounds of propolis is different based on the vegetation at the area from which it was collected (Bankova 2005). The main plant sources of propolis from temperate zones were poplar and birch tree (Roumen et al. 2006). On the other hand, conifer species of the Cupressaceae family were the main botanical source of

propolis from Mediterranean regions (Popova et al. 2012). The study of propolis from tropical rainforest climate especially Brazil showed that *Baccharis dracunculifolia* and *Dalbergia ecastaphyllum* were the main botanical origin (Dausghch 2008; Park 2004). Investigation of propolis from tropical Asia discovered *Macaranga tanarius* L. and *Mangifera indica* L. as the plant sources of the Indonesian propolis (Trusheva et al. 2011).

The main compounds of propolis from temperate zone (Europe, North America and non-tropic Asia) were flavonoids aglycones, phenolic acids and their esters (Maciejewicz et al. 2001; Hegazi et al. 2000). In contrast, the major compounds of propolis from tropical rainforest climate (Southeast Asia, Central Africa and South America) especially Brazil were artepillin C (Park et al. 2004) and prenylflavonones from Indonesian propolis (Trusheva et al. 2011). Whereas the main compounds of propolis from Mediterranean were terpenoids and sugars (Popova et al. 2011).

Literature survey showed that phenolic compounds, flavonoids and terpenoids were attributed to the biological activities of propolis. Propolis is well known to have various biological activities such as antibacterial (Boonsai et al. 2014), antifungal (Agüero et al. 2014), antiviral (Yildirim et al. 2016), anti-inflammatory (Machado et al. 2012; Naito et al. 2007), antioxidant (Ahn et al. 2007, 2004; Shiva et al. 2007), antitumour (Sobočanec et al. 2011; Xuan et al. 2014), hepatoprotective (Banskota et al. 2001; Paulino et al. 2014), neuroprotective (Nakajima et al. 2007; Shimazawa et al. 2005), anti-diabetic (Zhu et al. 2011), anti-amnesia (Chen et al. 2008), local anaesthesia (Paintz & Metzner 1979), antiulcer (de Barros et al. 2008) and UV light protection (Cole et al. 2010; Gregoris et al. 2011).

Malaysia is a tropical country blessed with wide range of plant species and hence the chemical constituents of Malaysian propolis should be different with propolis produced in other regions. Numerous studies on biological properties of Malaysian propolis investigated have revealed that propolis has the potential to assist in wound healing (Jacob et al. 2015), antifungal (Nik Yusliyana et al. 2016), antioxidant (Zayyanu Usman et al. 2016a) and antidiabetic (Zayyanu Usman et al. 2016b) activities. Few studies on chemical compounds in Malaysian propolis have been carried out which have shown a range of volatile and nonvolatile compounds (Nurhamizah et al. 2016; Nur Liyana et al. 2016; Zayyanu Usman et al. 2016a; Zayyanu Usman & Mahaneem 2015). However, these studies focused on propolis from stingless bees or *Trigona spp.* To date, no study has been reported on the analysis of chemical compounds in Malaysian propolis from *Apis mellifera* bees. Therefore, this study aimed to identify the various chemical constituents of propolis derived from *Apis mellifera* bees cultured in *Acacia mangium* apiary from northern and southern regions of Peninsular Malaysia.

## MATERIALS AND METHODS

### PROPOLIS SAMPLE

Two samples of raw *Apis mellifera* propolis, one from the northern and the other from the southern region of Peninsular Malaysia from *Acacia mangium* apiary were used in the current study. 250 g each of the pooled propolis samples collected from numerous beehives were supplied by the Malaysian Department of Agriculture, which were stored at -20°C until use.

### PREPARATION OF ETHANOLIC EXTRACT OF PROPOLIS

The extracts were prepared based on the methods described by Krell (1996) with some modifications. Propolis samples were cooled in a freezer (-20°C) for a day and were ground into a fine powder. Then, 50 g of each propolis sample was mixed with 167 mg of 70% ethanol to obtain 30% (w/w) propolis extract. The mixture was shaken twice a day and was left for a week at room temperature. Then, the mixture was filtered two times through Whatman filter paper No. 1. Prior to the second filtration, the extract was kept in refrigerator (2-8°C) in order to remove the wax. The ethanol was evaporated by using a rotary evaporator under vacuum at 35°C. The remaining water in the extract was dried by using freeze dryer and the dry extract was stored in the amber glass and was put in the freezer (-20°C).

### GC-MS ANALYSIS

#### SAMPLE PREPARATION FOR GC-MS

The dried extract (0.5 mg) was derivatised with 50 µl MSTFA in a sealed glass tube for 30 min at 60°C. The trimethylsilylated extract was cooled to room temperature before dilution with 50 µL of dodecane. The extract was transferred into crimped cap vial before GC-MS analysis. Blank ethanol (negative control) was dried and treated similarly as the sample.

#### GC-MS CONDITIONS AND PARAMETERS

GC-MS was carried out using Hewlett Packard 6890 Gas Chromatograph with 5973N Mass Selective Detector. The column was fused silica capillary, HP-5 column (30 m × 0.25 mm i.d × 0.25 µm film thickness) (Agilent Technologies, USA). The carrier gas was helium with flow rate at 1.0 mL/min with the oven temperature was programmed from 50°C (held for 2 min) to 280°C (held for 10 min) at a rate of 20°C/min. The injection and interface temperatures were set at 250°C and 280°C, respectively.

One microliter sample was injected in splitless mode and analysed in MS full scan mode ( $m/z$  40-650). The electron ionization was fixed at 70eV. Acquisition of data was performed using Chemsation software.

## IDENTIFICATION OF THE CHEMICAL CONSTITUENTS

Identification of chemical constituents was accomplished based on mass spectral matching with National Institute of Standards and Technology (NIST02) and Wiley275 libraries ( $\geq 80\%$  matching). The concentration of the identified compound was computed based on the percentage of the relative peak area (%).

## RESULTS AND DISCUSSION

The chemical constituents of EEP from northern and southern regions of Peninsular Malaysia, which were collected from *Acacia mangium* apiary, were investigated by GC-MS after silylation. Chemical compounds identified in this study include phenolic acids, fatty acids, terpenoids, sugars and their derivatives were similar with other propolis (Kartal et al. 2002).

Twenty-six individual compounds were identified and  $\beta$ -panasinsene was identified for the first time from propolis. Out of 26 chemical compounds, only 4 similar

compounds were identified in both regions. The chemical constituents of EEP as % of relative peak area are presented in Table 1. Although the propolis samples were collected from the same apiary, the difference of shrubs and fruit orchards around make the chemical compounds from two regions differs significantly.

The majority of identified compounds in EEP from northern and southern regions were terpenoids, which comprised of 11.46 and 7.19%, respectively. This finding was similar with *Apis mellifera* propolis from other tropic region (Li et al. 2009) and Mediterranean area (Popova et al. 2011, 2010). Similar finding was observed from *Trigona spp* propolis from Malaysia (Usman et al. 2016). However, the terpenoid compounds were different. Only one compound was similar, namely delta cardine. In the present study, eleven compounds of terpenoids were identified. Out of 11 identified terpenoids compounds, only 3 compounds were identified from both regions which are aromadendrene,  $\alpha$ -cedrol and globulol. The main terpenoids compounds identified in propolis from

TABLE 1. Chemical compounds of ethanolic extracts of Malaysian *Apis mellifera* propolis

Compounds	Relative peak area (%)	
	Northern region	Southern region
<i>Phenolic acids and esters</i>		
Gallic acid <sup>a</sup>	0.91	1.39
Cinnamic acid <sup>a</sup>		0.07
<i>Fatty acids and esters</i>		
Arachidic acid <sup>a</sup>	-	1.22
Behenic acid <sup>a</sup>	-	1.06
Ethyl linoleate <sup>a</sup>	-	0.20
Ethyl stearate <sup>a</sup>	-	0.12
Eicosanoic acid methyl ester <sup>a</sup>	0.57	-
<i>Terpenoids</i>		
Aromadendrene	1.15	0.03
$\alpha$ -cedrol	1.04	0.48
$\alpha$ -eudesmol	-	0.13
Lupenone	-	6.49
Globulol	2.51	0.06
$\gamma$ -gurjunene	1.68	-
$\delta$ -selinene	2.41	-
Ledene	1.47	-
$\delta$ -cadinine	0.14	-
$\beta$ -panasinsene	0.18	-
Epiglobulol	0.88	-
<i>Sugars and their Derivatives</i>		
Arabinofuranose <sup>a</sup>	-	0.49
D-fructose <sup>a</sup>	-	5.09
D-ribofuranose <sup>a</sup>	0.45	-
D-galactose <sup>a</sup>	-	0.18
Galacturonic acid <sup>a</sup>	0.97	-
Glycerol <sup>a</sup>	-	1.16
xylitol <sup>a</sup>	0.17	-
Erythritol <sup>a</sup>	-	0.83

<sup>a</sup> = Compound identified without TMS (trimethylsilyl) group

northern and southern regions were globulol and lupenone, respectively. Terpenoids are known to have antibacterial, antifungal (Melliou et al. 2007) and anticancer activities (Huang et al. 2012).

The other main class of propolis compounds was phenolic acids and their esters. The identified phenolic acids were gallic acid (3,4,5-trihydroxybenzoic acid) and cinnamic acid. Gallic acid was found in the propolis samples from both regions.

Fatty acids were the other compounds identified from propolis in the present study. Arachidic acid, behenic acid, ethyl linoleate, ethyl stearate, eicosanoic acid methyl ester were the identified fatty acids in Malaysian *Apis mellifera* propolis.

The other main class of propolis compounds was sugars. Arabinofuranose, D-fructose, D-ribofuranose, D-galactose, galacturonic acid, glycerol, xylitol and erythritol were the identified sugars from propolis samples in the current study. The amount of sugars was ranged from 1.59 to 7.76%. The high percentage of sugars indicates the plant source has high mucilage (Bankova et al. 2000). This finding was similar with propolis samples from Mediterranean (Popova et al. 2011, 2010) and tropic region in Asia; showed a significant amount of sugars and their derivatives (Syamsudin et al. 2009), range from 0.8 to 37.2%.

Flavonoids were not identified from Malaysian propolis which was consistent with the findings of GC-MS analysis of propolis from tropic regions (Syamsudin et al. 2009) and Mediterranean regions (Popova et al. 2011, 2010) most probably due to their plant source. *Acacia mangium* and rainforest tree nearby might be the main plant source of propolis in the current study; while the main plant source of European propolis were poplar trees (Roumen et al. 2006; Toreti et al. 2013). Rainforest trees are rich in glycoside flavonoid which is very polar and might be not amenable to GC-MS, whereas poplar tree is rich in non-polar flavonoids aglycones. In contrast, other studies on Malaysian propolis showed the presence of flavonoids. However, the propolis samples were from *Trigona spp* bees and were analysed by thin layer chromatography (Nurhamizah et al. 2016) and Folin-Ciocalteu method (Zayyanu Usman et al. 2016b). The difference could be due to the bee species or analytical technique.

There are several methods to identify and quantify the chemical constituents of propolis. The method chosen are depending on the chemical nature of the interested propolis compounds. To identify the compounds, the mixture will be separated first, and then the compounds will be identified. GC is one of the great separation techniques using carrier gas as mobile phase. It is very effective, sensitive (can detect ppm), require small sample ( $\mu$ l), accurate, fast analysis (typically minutes), quantitative and well known. A part of that, GC-MS has the advantages of higher chromatographic resolution and higher peak capacity, a single mobile phase (helium), fewer issues with solubility and separations that can be adjusted by electronic controls such as temperature and flow programming (Hübschmann 2015).

However, GC has few weaknesses such as the sample must be volatile, heat-stable and need experience and training (McNair & Miller 2011). The limitations of GC can be overcome by derivatising the samples to increase their volatility and thermal stability. In the current study, propolis extract was derivatised by using silylation reaction. Mass spectrometer is the most widely used detector for GC in propolis analysis. Identification of compounds is based on mass spectrum. Mass spectrum produced by a given chemical compound is basically the same every time. Therefore, the mass spectrum is essentially a fingerprint for the compounds. The mass spectrum of the each compound can be compared by mass spectra library to identify unknown compounds.

Many studies have analysed the propolis compounds using GC-MS (Murat 2002; Maciejewicz et al. 2001; Park et al. 2002) and HPLC methods (Osmany et al. 2007; Medana et al. 2008; Volpi & Bergonzini 2006) since the propolis compounds has varied polarity.

Ideally, non-polar or volatile compounds are easier to analyse by GC-MS, whereas for identification of polar or non-volatile compounds, HPLC is better. However, derivatisation can increase the volatility and polarity of the compounds, thus make the compounds amenable to GC-MS analysis. Compared to HPLC, GC-MS is more potent and providing a general view of propolis compounds (Garcia-Viguera et al. 1993; Marquez Hernandez et al. 2010). GC-MS analysis of propolis showed this technique able to identify flavonoids, phenolic acid and their esters, aromatic compounds, fatty acids, terpenoids, essential oils and sugars. However, for the specific analysis of the phenolic compounds, HPLC with PDA is more useful (Garcia-Viguera et al. 1993). Therefore, GC-MS technique was chosen to identify the overall view of propolis in this research since of the study on *Apis mellifera* propolis from Malaysia is still lacking. Further, quantitative analysis for each identified compound is needed to establish the criteria for the quality control of national propolis samples.

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